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Lipopolysaccharide levels are elevated in dengue virus infected patients and correlate with disease severity

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ABSTRACT

Background: Although in the majority of cases dengue virus (DENV) infection results in a self-limiting febrile disease, it can cause severe plasma leakage in a minority of patients. The appearance of plasma leakage indicates an increased permeability of the vascular wall. In this study we investigated if DENV infection can lead to leakage of lipopolysaccharide (LPS) from the intestine into the blood of the patient, indicative of an increased permeability of the intestinal mucosal barrier.
Objectives: The aim of this study was to investigate if LPS levels were elevated in DENV infected patients and if these levels correlated with disease severity.
Study design: LPS levels in the blood of DENV infected children were determined using the Limulus Amebocyte Lysate assay. To determine disease severity we used the 1997-WHO criteria, the expert physician's judgement and a score that focused on plasma leakage in particular. Furthermore, the modulatory factors LPS binding protein (LBP) and sCD14, as well as the immune activation marker neopterin were determined.
Results: We showed significantly elevated LPS levels in plasma of DENV infected children compared to healthy controls. The plasma leakage severity score had the strongest correlation with levels of LPS. LBP, sCD14 and neopterin were elevated compared to healthy controls.
Conclusion: In this study we show evidence of elevated LPS levels during DENV infection. Moreover, a correlation between LPS levels and disease severity was found, especially when disease severity was determined in terms of plasma leakage.

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1. Background

Dengue virus (DENV) belongs to the family *Flaviviridae* and consists of four serotypes (DENV 1–4). Annually an estimated number of 50–100 million infections occur in tropical and subtropical countries around the world. DENV-infection usually results in a subclinical or self-limiting febrile disease but may also lead to severe disease, previously known as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Severe disease is characterised by thrombocytopenia, haemorrhagic manifestations, liver disturbances and a sudden onset of vascular permeability, believed to be caused by a cytokine storm (reviewed in[1]).

The primary target cells of DENV are monocytes and tissue macrophages. In humans the gut-associated lymphoid tissue (GALT) contains the largest pool of macrophages and memory T cells. Another virus that targets mononuclear cells is human immunodeficiency virus (HIV). Recently it was shown that replication of HIV in the GALT was associated with elevated LPS levels, also known as microbial translocation (MT).[2]

MT and its effects have extensively been studied in diseases where the intestinal mucosal barrier is severely damaged or inflamed, e.g. in graft vs. host disease after hematopoietic stem cell transplantation[3] and inflammatory bowel disease.[4] In these diseases it has been found that MT contributes significantly to the inflammatory response. Moreover, it has been shown that in Gram-negative sepsis, a disease with generalized vascular permeability

Abbreviations: DENV, (dengue virus); LPS, (lipopolysaccharide); MT, (microbial translocation); LBP, (lipopolysaccharide binding protein); sCD14, (soluble CD14); mCD14, (membrane-bound CD14); GALT, (gut-associated lymphoid tissue); HIV, (human immunodeficiency virus); DF, (dengue fever); DHF, (dengue haemorrhagic fever); DSS, (dengue shock syndrome); PLS, (plasma leakage severity); LAL, (Limulus Amebocyte Lysate).

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caused by an exaggerated immune response, MT occurs and correlates with disease severity and clinical outcome.[5]

Like sepsis, the hallmarks of severe DENV infection are plasma leakage and a highly activated immune system.[1] Moreover, like HIV DENV predominantly replicates in mononuclear cells of which many reside in the GALT. Taken together, we hypothesize that DENV-infection may cause MT and that LPS levels may correlate with disease severity.

1.1. Objectives

The objective of this study was to investigate whether elevated LPS levels can be found in DENV infected patients and whether these levels correlate with disease severity.

2. Study design

2.1. Clinical cohort

Following written parental consent, serial blood samples from 200 children aged 3–14 years with a clinical suspicion on DENV infection were collected at the Dr. Kariadi Hospital in Semarang, Indonesia in the period of February 2001 till March 2003.[6] This study was approved by the ethics committee of the Dr. Kariadi Hospital.

DENV infection was confirmed by serotype-specific reverse transcription-PCR (RT-PCR) from the samples obtained at day of admission[7] or by detection of DENV specific IgM antibodies (Focus technologies) in acute and/or convalescent samples. Thirty-seven children from the same area who had the same genetic background but a negative serology for DENV-infection served as healthy controls.

Patients were classified using three classification systems. The first two were the stringent 1997-WHO-criteria[8] and the expert physician's judgement of disease severity (MD classification). The MD classification is the diagnosis given by the treating physician and recorded in the patient's chart. The possible diagnoses in the MD classification are: DF, DHF and DSS – intuitively based on physical examination, clinical course and laboratory data.

The third classification system was based on a plasma leakage severity (PLS) score that takes the following signs into account: Ht > 45.0%, tachycardia (pulse >120/min), hypotension (systolic blood pressure <80 mmHg), narrow pulse pressure (difference between systolic and diastolic blood pressure <20 mmHg), pleural effusion (proven by X-ray), and signs of ascites (shown by physical examination). Each sign present is assigned a value 1; the PLS score was defined as the sum of these values, ranging from 0 to 6. Haemoglobin and haematocrit levels, as well as any signs of plasma leakage were recorded daily.

2.2. Laboratory determinations

Samples have been stored at −80° C and repetitive freeze–thaw cycles have been avoided. LPS was determined with a commercially available Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod Incorporated). Because components in plasma may interfere with this assay,[9] plasma samples were diluted 1:5 with LAL reagent water and heat-inactivated at 60 °C for 30 min. Known concentrations of *Escherichia coli* LPS were diluted in heat-inactivated negative plasma (1:5 dilution) and served as standard values. The standard and plasma samples were incubated with the pyrochrome for 70 min at 37 °C. After the reaction was stopped the test was read at an absorbence of 405 nm.

Soluble CD14 (sCD14; 'Quantikine' ELISA, R&D Systems), LPS binding protein (LBP; HK315′ ELISA, Hycult Biotech), and neopterin (IBL ImmunoBiological Laboratories) were determined using commercially available assays. To this end, samples were diluted 1:200 (sCD14), 1:1000 (LBP), and used undiluted (neopterin). The assays were performed according to the manufacturer's instructions.

2.3. Statistical analysis

The Kruskal Wallis *H* test was used for comparison of more than two groups. Statistical significance between individual groups was determined with the Mann Whitney *U* test. Correlations between continuous variables were determined with the Spearman correlation coefficient test. *P* values ≤0.05 were considered significant.

3. Results

Eighty-nine plasma samples from Indonesian children with laboratory confirmed DENV infection were selected for this analysis. Clinical characteristics of the cohort are summarized in Table 1. Based on the MD classification, more patients had severe disease (DHF/DSS) than those diagnosed with the 1997-WHO classification (Table 1). The application of the PLS score to both classifications supported the MD classification of disease severity. The median PLS score of patients classified with DF, DHF and DSS according to the

Table 1
Patient characteristics of the Indonesian cohort classified according to the 1997-WHO criteria and the physician's judgement (MD diagnosis). Age, days of fever, Hb, Ht and plasma leakage severity score are expressed as median (interquartile range).

Classification	WHO classification			MD classification		
	DF *N* = 18	DHF *N* = 45	DSS *N* = 26	DF *N* = 10	DHF *N* = 35	DSS *N* = 44
Sex	50.0% male	51.1% male	46.2% male	50.0% male	48.6% male	50.0% male
Age (years)	7.5 (4–11)	8 (5–10)	7 (5–10)	8 (6.25–11.5)	8 (6–10)	7 (5–10)
Days of fever	3 (2–3.25)	3 (3–4)	4 (3.75–5)	3 (2–4)	4 (3–4)	4 (3–4)
Haemoglobin (g/dl)	11.8 (10.9–13.1)	13.2 (12.0–14.3)	14.1 (12.2–14.9)	11.9 (11.4–13.1)	12.9 (12.0–13.9)	13.8 (11.9–15.1)
Haematocrit (%)	35.7 (23.1–36.7)	40.9 (36.9–43.6)	43.0 (40.4–47.9)	36.7 (33.3–40.0)	40.7 (36.8–43.0)	42.5 (37.2–47.0)
Tachycardia (pulse >120/min)	22.2%	31.1%	65.4%	10.0%	28.6%	54.5%
	N = 4	*N* = 14	*N* = 17	*N* = 1	*N* = 10	*N* = 24
Hypotension (systolic blood pressure <80 mmHg)	22.2%	0%	46.2%	10.0%	0%	34.1%
	N = 4	*N* = 0	*N* = 12	*N* = 1	*N* = 0	*N* = 15
Narrow pulse pressure (diff <20 mmHg)	16.7%	0%	23.1%	0%	0%	20.5%
	N = 3	*N* = 0	*N* = 6	*N* = 0	*N* = 0	*N* = 9
Pleural effusion (X-ray)	38.9%	40.0%	80.8%	0%	28.6%	81.8%
	N = 7	*N* = 18	*N* = 21	*N* = 0	*N* = 10	*N* = 36
Ascites (physical examination)	0%	2.2%	3.8%	0%	0%	4.5%
	N = 0	*N* = 1	*N* = 1	*N* = 0	*N* = 0	*N* = 2
Plasma leakage severity score	1 (0–2)	1 (0–1)	2 (2–3.25)	0 (0–0.25)	1 (0–1)	2 (2–3)

WHO system was 1, 1, and 2 respectively. In contrast, the median PLS score in the MD classification for DF, DHF, and DSS was 0, 1 and 2 respectively. It has been reported previously[10] that due to the stringency of the 1997-WHO classification patients with severe disease according to the expert physician's judgement may be classified as having non-severe disease (Table 1).

To investigate whether MT occurred during DENV infections, LPS levels were measured. Significantly elevated LPS levels were detected in the plasma of all DENV infected patients compared to healthy controls (Fig. 1A–C). LPS levels were subsequently analyzed in relation to disease severity. Using the 1997-WHO classification only a significant difference was found between DHF and DSS patients ($P = 0.04$) (Fig. 1A). However, when patients were divided according to the MD classification, LPS levels in patients with DSS differed significantly from those with DHF ($P < 0.001$) and DF ($P = 0.01$).

When plasma LPS levels were compared in patients classified according to the PLS score, a significant difference was observed between the group with no signs and the groups with three and more signs ($P = 0.006$) (Fig. 1C). A trend was shown in which a higher PLS score correlated with increased LPS levels.

As LBP and sCD14 play an important role in the modulation of the LPS response we measured their levels in relation to the PLS score. As shown in Fig. 1D, the LBP-levels in the group with three and more symptoms were significantly lower than the levels in groups with no symptoms ($P = 0.002$). The levels of the healthy controls were significantly lower than in all other groups ($P < 0.0001$). Similarly, the levels of sCD14 in the group with three and more symptoms were slightly lower than the group with no symptoms ($P = 0.046$) (Fig. 1E). The levels in all groups were elevated compared to the healthy controls ($P < 0.001$).

To investigate whether MT was associated with activation of monocytes/macrophages the levels of neopterin were determined. The elevated neopterin levels indicated that monocytes/macrophages were significantly activated in DENV infected patients compared to healthy controls ($P \leq 0.01$). Consistently, a

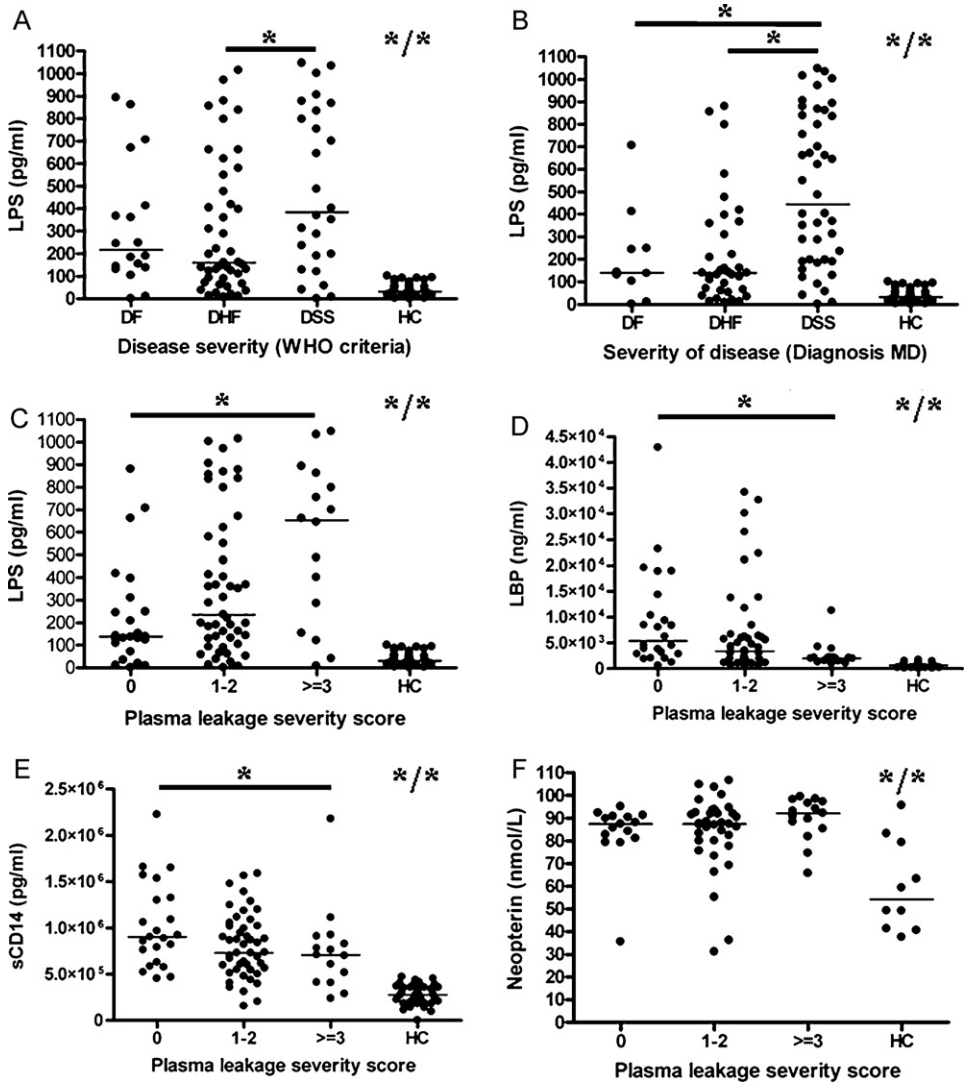

Fig. 1. LPS, LBP, sCD14 and neopterin in relation to severity of disease. (A) Correlation between LPS levels and severity of disease based on 1997-WHO criteria. Significant differences were found for DHF vs. DSS ($P = 0.04$); DF, DHF and DSS vs. healthy controls (HC) (all $P < 0.0001$). (B) Correlation between LPS levels and severity of disease based on MD classification. Significant differences for DF vs. DSS ($P = 0.01$); DHF vs. DSS ($P = 0.0001$); DF vs. HC ($P = 0.003$); DHF and DSS vs. HC ($P < 0.0001$). (C) Correlation between LPS levels and severity of disease based on plasma leakage severity (PLS) score. Significant differences for 0 vs. ≥3 ($P = 0.006$); 0, 1–2 and ≥3 vs. HC ($P < 0.0001$). (D) Correlation between LBP levels and severity of disease based on PLS score. Significant differences for 0 vs. ≥3 ($P = 0.002$); 0, 1–2 and ≥3 vs. HC ($P < 0.0001$). (E) Correlation between sCD14 levels and severity of disease based on PLS score. Significant differences for 0 vs. ≥3 ($P = 0.046$); 0, 1–2 and ≥3 vs. HC ($P < 0.0001$). (F) Correlation between neopterin and severity of disease based on PLS score. Significant differences for 0 vs. HC ($P = 0.001$); 1–2 vs. HC ($P = 0.003$); ≥3 vs. HC ($P = 0.001$). Horizontal lines indicate the median of the groups. ___*___ means significant difference between two groups.*/* means that this group is significantly different from all other groups.

weak correlation between levels of LPS and levels of neopterin was observed ($P = 0.023$, $r = 0.29$) (data not shown). Moreover, there was a slightly stronger correlation between LBP and sCD14-levels ($P = 0.0002$, $r = 0.40$) (data not shown).

4. Discussion

In this study we found elevated levels of LPS in DENV-infected patients compared to healthy controls. Moreover, the levels of LPS showed to have a correlation with disease severity, indicated by an increased PLS score.

Classifying DENV-infected patient in terms of disease severity has always been a challenge to physicians and researchers in the field. The application of our PLS score to the 1997-WHO classification and the MD classification suggests that the MD classification reflects disease severity more accurately.

Plasma leakage is a hallmark of severe DENV-infection and may be indicative of increased permeability of the vascular wall. Therefore we hypothesized that a similar mechanism causing plasma leakage may be in part responsible for MT. In agreement with this the PLS score showed the best correlation with LPS levels compared with the 1997-WHO and MD-classification. However, the PLS score only accounts for plasma leakage and shock, while haemorrhagic, gastrointestinal and liver manifestations are not taken into account. Nevertheless, LPS levels did not correlate with the occurrence of haemorrhage and the levels of liver enzymes (data not shown). From the gastrointestinal manifestations diarrhea is most likely to be associated with MT. However, only four patients were reported to suffer from diarrhea in our study population.

Taken together, our data suggest that in order to investigate certain specific pathogenetic pathways in humans it might be useful to classify patients according to specific clinical symptoms and laboratory markers.

Because of the interference with plasma components the LAL assay is known to be very challenging and therefore several strategies have been described.[9] Interestingly, the absolute values of LPS in our study were higher compared to those reported in other studies.[2,4,5] The difference between this study and others is that the standard was diluted in a plasma dilution comparable with the dilution of the patient samples. Because of the inhibitory activity of plasma the standard should contain the same amount of plasma as the diluted samples.

LPS is an important immune stimulator, because it can activate the innate immune system by signalling through a membrane-bound CD14(mCD14)/Toll-like receptor 4 complex. The systemic response upon the release of LPS in the circulation is modulated by LBP and sCD14. LBP is synthesized by the liver upon stimulation with LPS and can accelerate the transfer of LPS to membrane-bound CD14. On the other hand LBP can also shuttle LPS to lipoproteins.[11] The LBP concentrations in patients with a PLS score ≥ 3 were very low, suggesting that severely ill patients fail to produce enough LBP to neutralize LPS. These results are comparable with those from studies about MT and septic shock, in which non-survivors had lower LBP values than survivors.[5]

sCD14 is produced by monocytes upon activation by LPS and has been suggested as a marker for disease severity.[12] In our study population the levels of sCD14 were significantly elevated in all groups compared to healthy controls. Moreover, the group with the highest PLS score also showed decreased levels of sCD14, but to a lesser extent than the LBP levels. This was also found in patients with sepsis.[13] A dual mechanism may be involved here. On the one hand, high concentrations of sCD14 prevent an exaggerated immune response by competing with LPS for mCD14. On the other hand, low amounts of sCD14-LPS complexes can activate endothelial cells.[14]

In HIV infected individuals MT has also been found and shown to correlate with the activity of the disease.[2] During active HIV disease infection and depletion of CD4 + T cells contributes significantly to disease pathogenesis. A substantial part of these CD4 + T cells reside in the GALT and therefore the GALT is probably highly affected in these individuals, which eventually can result in impaired mucosal integrity.[15] It is not clear whether DENV replicates in cells of the GALT. However, it is likely because intestinal macrophages represent the largest pool of tissue macrophages in humans. We hypothesize that DENV replication in intestinal macrophages may lead to a pro-inflammatory environment in which the epithelial cells are disrupted. In agreement with other studies[16,17] we found increased levels of neopterin in DENV-infected patients indicative of monocyte activation.

Taken together, this is the first study suggesting that DENV infection may be associated with MT, as measured by plasma levels of LPS. Furthermore, levels of LPS seemed to correlate with disease severity. Further studies are needed to elucidate the implications of this finding.

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Competing of interest

None of the authors declare conflict of interest apart from Albert Osterhaus who is a part time employee of Viroclinics BV (for details go to www.erasmusmc.nl). The stated competing interest does not alter the author's adherence to the policies on sharing data and materials.

Ethical approval

This study was approved by the ethics committee of the Dr. Kariadi Hospital.

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